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Faassen, Martijn van; Bischoff, Rainer; Kema, Ido P

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Martijn van Faassen*, Rainer Bischoff and Ido P. Kema

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Abstract

Background: Disturbance of the circadian rhythm has been associated with disease states, such as metabolic disorders, depression and cancer. Quantification of the circadian markers such as melatonin and cortisol critically depend on reliable and reproducible analytical methods. Previously, melatonin and cortisol were primarily analyzed separately, mainly using immunoassays.
Methods: Here we describe the validation and application of a high-throughput liquid chromatography in combination with mass spectrometry (LC-MS/MS) method for the combined analysis of melatonin and cortisol in plasma and saliva. The LC-MS/MS method was validated according to international validation guidelines. We used this method to analyze total plasma, free plasma (as obtained by equilibrium dialysis) and saliva melatonin and cortisol in healthy adults.
Results: Validation results for plasma and saliva melatonin and cortisol were well within the international validation criteria. We observed no difference between saliva collected by passive drooling or Salivette. Moreover, we noted a significant difference in saliva vs. free plasma melatonin. We observed on average 36% (95% CI: 4%–60%) higher salivary melatonin levels in comparison to free plasma melatonin, suggestive of local production of melatonin in the salivary glands.
Conclusions: The novel outcome of this study is probably due to the high precision of our LC-MS/MS assay. These outcomes illustrate the added value of accurate and sensitive mass spectrometry based methods for the quantification of neuroendocrine biomarkers.

***Corresponding author: Martijn van Faassen,** BASc, Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, EA61, P.O. Box 30.001, 9700 RB Groningen, The Netherlands, Phone: +31 50 361 03 99, Fax: +31 50 361 91 91, E-mail: h.j.r.van.faassen@umcg.nl
Rainer Bischoff: Analytical Biochemistry, Department of Pharmacy, University of Groningen, Groningen, The Netherlands
Ido P. Kema: Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Keywords: cortisol; dialysis; liquid chromatography in combination with mass spectrometry (LC-MS/MS); melatonin; plasma; saliva.

Introduction

There is mounting evidence that disturbance of circadian rhythm is involved in the etiology of several disease states such as depression, the metabolic syndrome and cancer [1, 2]. Typical circadian rhythm markers are melatonin and cortisol. Melatonin, a pineal hormone and metabolite of the tryptophan-serotonin-pathway is best known for its role as a signaling molecule for the length of day and night [3, 4]. Cortisol, an adrenal cortex derived glucocorticoid hormone, is at the end-point of the hypothalamic-pituitary-adrenal axis, and adapts the body to stress conditions by mobilizing energy and inhibiting non-emergency processes, such as sleep, sexual activity and growth [5]. The cortisol response is further related to awakening, as cortisol peaks after waking up [6].

Quantification of melatonin is still mainly performed by immunochemical methods, whereas in recent years cortisol determination has shifted towards liquid chromatography in combination with mass spectrometry (LC-MS/MS). This discrepancy in the methods used is probably due to the fact that cortisol is a frequently used diagnostic marker in routine patient care (for Cushing's syndrome) [7, 8]. While a few reports have been published on melatonin analysis in saliva or plasma by LC-MS/MS, validation was not performed according to internationally accepted guidelines [9–12]. When compared to immunoassays LC-MS/MS has significant advantages due its better analytical reproducibility, specificity and standardization. Immunoassays tend to lack specificity and show variability between vendors [13–16]. Moreover, LC-MS/MS has the possibility of measuring multiple analytes in one assay, whereas immunoassays are mainly single-analyte assays. In circadian rhythm studies measurements of melatonin and cortisol are often performed separately, where it would be more effective to combine measurements of these analytes thereby saving sample, time and costs [17–20].

Circadian rhythm studies usually have multiple sampling points on consecutive days and therefore saliva is

preferred, as its sampling is noninvasive and more practical than blood sampling. Another advantage of saliva is, that it reflects the free non-protein bound fraction of plasma, which is considered the biologically active fraction [21]. For cortisol, the relationship between saliva and plasma free cortisol plasma has been extensively studied, and late night salivary cortisol is now routinely used as diagnostic test for Cushing's syndrome [22–24]. However, to our knowledge there is only one study which describes the relationship between total plasma, free plasma, and salivary melatonin, which uses an immunoassay [25]. In this study, we investigated the correlations between total plasma, free plasma and salivary cortisol and melatonin levels by a fully validated LC-MS/MS method and show for the first time a significant difference in saliva vs. free plasma melatonin.

Participants and methods

Materials

Water, acetonitrile, methanol, formic acid and ammonium acetate were obtained from Biosolve and were ULC/MS grade. Ascorbic acid was purchased from Merck. Isopropanol, cortisol, melatonin, cortisone, prednisone and prednisolone were purchased from Sigma-Aldrich. Deuterated analogs of cortisol (d4) and melatonin (d4) were purchased from C/D/N Isotopes.

Sample collection and preparation

Morning blood and saliva samples (8:00 AM) were collected simultaneously in 10 non-smoking volunteers. Saliva was collected in two ways, either by passive drooling into polypropylene tubes or by cortisol Salivettes® (Sarstedt, Nümbrecht, Germany). Participants did not eat or drink 30 min before saliva sampling. Salivette rolls were kept in the mouth for 1 min and centrifuged at 1000 *g* for 2 min. One milliliter of saliva of passive drooling and Salivette was collected and stored at −20 °C until analysis. Before analysis, saliva was centrifuged for 5 min at 2000 g. Blood samples were taken via venipuncture, using 4 mL Vacutainer Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing K_2-EDTA as anticoagulant. Blood samples were centrifuged at 2500 *g* for 15 min and plasma was stored at −20 °C until analysis. Free melatonin and cortisol were determined in plasma dialysate obtained by equilibrium dialysis essentially as described by Fiers et al. for testosterone [26], with the exception that the dialysis membranes used in our study had a 10 kDa pore size instead of 25 kDa pore size, as cortisol binding globulin is significantly lower in molecular weight than sex hormone binding globulin, 50 and 90 kDa, respectively.

The study was evaluated by the Local Medical Ethics Committee and waived, because the purpose of this study was to compare melatonin and cortisol levels in saliva to the values in plasma and the burden for the participants was considered to be very low. All participants gave written informed consent.

Analytical procedures

Stock standards for melatonin and cortisol were prepared in methanol at a concentration of 1.0 g/L (melatonin 4.31 mmol/L, cortisol 2.76 mmol/L). Eight individual calibrators were prepared fresh from the stock solution in 0.04% (v/v) ascorbic acid on the day of analysis. Calibration curves for saliva were prepared in 0.04% ascorbic acid and for plasma in blank plasma. Plasma depleted of melatonin and cortisol was prepared by extensive dialyzing plasma against PBS using a 10 kDa molecular weight cut-off membrane.

For saliva the calibrator ranges were: melatonin 15–2000 pmol/L, and cortisol 0.60–75 nmol/L. For plasma: melatonin 15–2000 pmol/L, and cortisol 40–5500 nmol/L. Internal standard working solutions for saliva (melatonin 800 pmol/L, cortisol 100 nmol/L) and plasma (melatonin 800 pmol/L, cortisol 1000 nmol/L) were prepared on the day of analysis in 0.04% (v/v) ascorbic acid. Quality control samples were prepared by pooling several saliva or plasma samples and were, when necessary, spiked with melatonin and cortisol to give three pools (low, medium and high). All samples were stored at −70 °C until use.

For analysis, 250 µL saliva/dialysate, 50 µL internal standard solution and 200 µL 0.04% ascorbic acid were added to wells of a 2.0 mL polypropylene 96-deep well plate (Greiner Bio-One). The plate was sealed before being vortex mixed for 10 min. Following vortexing, the plate was transferred to the autosampler and 100 µL was injected for analysis.

For plasma analysis, 200 µL of plasma was pipetted into a 2.0 mL 96-deep well plate (Greiner Bio-One). To this, 50 µL of internal standard solution was added, and the plate was vortex mixed for 1 min. Proteins were precipitated by adding 200 µL of 0.3 M zinc sulfate in methanol (1 : 5 v/v) and the plate was vortexed again for 1 min. Water was added to fill up to 1.0 mL and the plate was sealed and centrifuged for 15 min at 2500 × *g*. Following centrifugation, the plate was transferred to the autosampler and 100 µL of the supernatant was injected for analysis.

Online solid phase extraction and LC-MS/MS

Online solid phase extraction (SPE) was performed using the fully automated Symbiosis™ system (Spark Holland, Emmen, The Netherlands) in eXtraction liquid chromatography (XLC) mode as previously described [27]. Oasis HLB (Waters, Milford, USA) cartridges were used for extraction. Liquid chromatography (LC) was performed on a Phenomenex® Luna Phenyl-Hexyl 2.0×100 mm, 3 µm column (Phenomenex, Torrance, CA, USA) and total run time was 6.5 min, including online SPE cleanup. For a complete description of the online SPE and LC method see Supplemental Data. Melatonin and cortisol were analyzed in positive ionization mode on a Waters® Xevo™ TQ-MS triple quadrupole mass spectrometer. Mass spectrometer settings were optimized by tuning in the selective reaction monitoring mode (SRM). The following settings were applied throughout the study: capillary voltage 0.5 kV, desolvation temperature 600 °C, desolvation gas flow 1000 L/h, cone gas flow 50 L/h and collision gas flow 0.15 mL/min. Cone voltage and collision energies were optimized for all transitions and are listed in Table 1. Quantifier and qualifier *m/z* transitions were monitored for melatonin and cortisol as well as for their internal standards melatonin-d4 and cortisol-d4. Quantitation was performed by using the peak-area

Table 1: Transitions, cone voltage and collision energy for quantifier and qualifier of each compound.

Compound	*m/z*	Cone voltage, V	Collision energy, eV
Melatonin-quantifier	233.2 > 174.1	18	14
Melatonin-d4	237.2 > 178.1	18	14
Melatonin-qualifier	233.2 > 159.1	18	27
Melatonin-d4	237.2 > 163.1	18	27
Cortisol-quantifier	363.3 > 121.1	25	23
Cortisol-d4	367.3 > 121.1	25	23
Cortisol-qualifier	363.3 > 327.3	25	16
Cortisol-d4	363.3 > 331.1	25	16

response ratios of the quantifier transitions for cortisol : cortisol-d4 and melatonin : melatonin-d4 using the Targetlynx™ software.

Analytical validation

Method validation was performed for saliva and plasma to the same extent, unless otherwise stated and based on the guideline for bioanalytical method validation from the European Medicines Agency [28].

Linearity of the calibration curves for melatonin and cortisol was assessed by analyzing the calibration curves on 12 different days. Curves were plotted using least-squares linear regression and checked for linearity. To test whether melatonin and cortisol behaved the same in water-based calibrators as in saliva-based calibrators, we spiked calibration curves in six different saliva samples to compare the slope in comparison to water-based calibrators.

Intra-assay variation was determined by analyzing three pooled saliva or plasma samples with melatonin and cortisol at low, medium and high concentrations in 20 replicates. Inter-assay variation was determined by analyzing three pooled saliva or plasma samples on 12 different days in duplicate over an 8-week period.

Quantification limits for melatonin and cortisol were determined by serial dilution of a saliva sample (melatonin 20 pmol/L, cortisol 0.4 nmol/L,) and plasma sample (melatonin 20 pmol/L, cortisol 130 nmol/L) and analyzing the dilutions on six different days in duplicate (for plasma on three different days). LLOQ was set where the precision was < 20% and the signal to noise ratio > 10.

Recovery was estimated by spiking melatonin and cortisol at three different levels to three different pooled saliva and plasma samples containing endogenous melatonin and cortisol at three different concentration levels. These samples were analyzed on six different days (for plasma on three different days). Recovery percentage was calculated as follows: [(final concentration − initial concentration)/added concentration] × 100%. Recovery was considered acceptable between 85 and 115%.

Ion suppression was checked by performing a post-column infusion experiment [29]. Solvent, saliva and plasma samples (n = 2) containing a low concentration of melatonin and cortisol were analyzed as described above with constant post-column infusion of melatonin and cortisol at a flow-rate of 10 µL/min (melatonin and melatonin-d_4 at 800 pmol/L, cortisol, cortisol-d_4 at 20 nmol/L). Chromatograms of the samples were compared with those of the solvent blank.

Cortisone, prednisone and prednisolone are known compounds that can interfere with cortisol analysis. These compounds were analyzed at a concentration of 100 nmol/L and checked for interference in the cortisol analysis. For melatonin, n-acetylserotonin, 6-hydroxymelatonin and 6-sulfatoxymelatonin were analyzed at a concentration of 100 nmol/L and checked for interference in the melatonin analysis.

Method comparison

Thirty-five anonymized saliva samples, which were analyzed in routine patient care for cortisol by an existing XLC-MS/MS method, were also analyzed by the new method. This routine XLC-MS/MS method was developed in our lab based on the method described by Jones et al. using C18 cartridges for online SPE and a 3.0 × 150 mm phenylhexyl column [30]. For salivary melatonin, we compared our method to a commercial immunoassay from IBL International (Hamburg, Germany) using the same saliva samples as mentioned above. Bland-Altman and Passing-Bablok regression were used for evaluation of the results using RStudio [31–33]. Ninety-five percent confidence intervals (CI) of slope and intercept were calculated to investigate if there was a significant difference (CI of slope should contain 1, CI of intercept should contain 0).

Impact of collection device and correlation between plasma total, plasma free and salivary melatonin and cortisol

Saliva was collected by passive drooling (PD) and by Salivette (SAL), and plasma from 10 healthy volunteers (six females and four males, with respective mean age of 31 and 35 years) to investigate the potential influence of the collection device on cortisol and/or melatonin. Furthermore, we analyzed total and free plasma, and salivary melatonin and cortisol to investigate the correlations in these matrices.

Statistics

Statistical analyses were performed using RStudio [34]. Non-parametric correlations (Spearman's ρ) were calculated to investigate the relationship between total plasma free plasma, and saliva melatonin and cortisol. A two-sided $p < 0.05$ was considered statistically significant. Passing-Bablok regression analysis was performed to examine the distribution of melatonin and cortisol between the matrices. Ninety-five percent confidence intervals (CI) of slope and intercept were calculated to investigate if there was a significant difference (CI of slope should contain 1, CI of intercept should contain 0).

Results

Assay performance

Total analysis time including automated sample extraction using the online SPE step was 6.5 min. Representative chromatograms obtained for saliva, plasma and plasma

dialysate are presented in Figure 1. Chromatographic selectivity was achieved by using a phenyl-hexyl column which baseline separates cortisone from cortisol and minimizes interference from prednisone (0%) and prednisolone (0.4% at 100 nmol/L prednisolone) while maintaining short run times. The analytes n-acetylserotonin, 6-hydroxymelatonin and 6-sulfatoxymelatonin did not show any interference in the melatonin assay. Melatonin and cortisol were not baseline separated, but this causes no erroneous results as detection is based on different mass over charge ratios of the precursor ions and the product ions. Melatonin eluted first at 3.26 min, followed by cortisol at 3.37 min.

Method validation

Calibration curves were linear over the calibration range for both melatonin and cortisol over n = 12 days with correlation coefficients, $R^2 > 0.99$. Intra-assay CV was below 9% for cortisol and below 6% for melatonin at all three QC levels for saliva and plasma. Inter-assay CV was below 10% for cortisol and below 9% for melatonin at the three QC levels (see Table 2). Complete results for the intra- and inter-assay variation of the method for saliva and plasma are shown in Table 2. The lower limit of quantification (LLOQ) was determined to be 4.0 pmol/L for melatonin, and 0.1 nmol/L for cortisol in saliva and 10 pmol/L, 0.5 nmol/L in plasma for, respectively, melatonin and cortisol. Recoveries for melatonin at the three levels ranged from 87% to 98% and from 97% to 111% for cortisol (see Table 3). No significant ion suppression was present at the retention times where melatonin and cortisol eluted.

Method comparison

In order to verify our method for salivary cortisol, we compared it to the currently used routine LC-MS/MS method

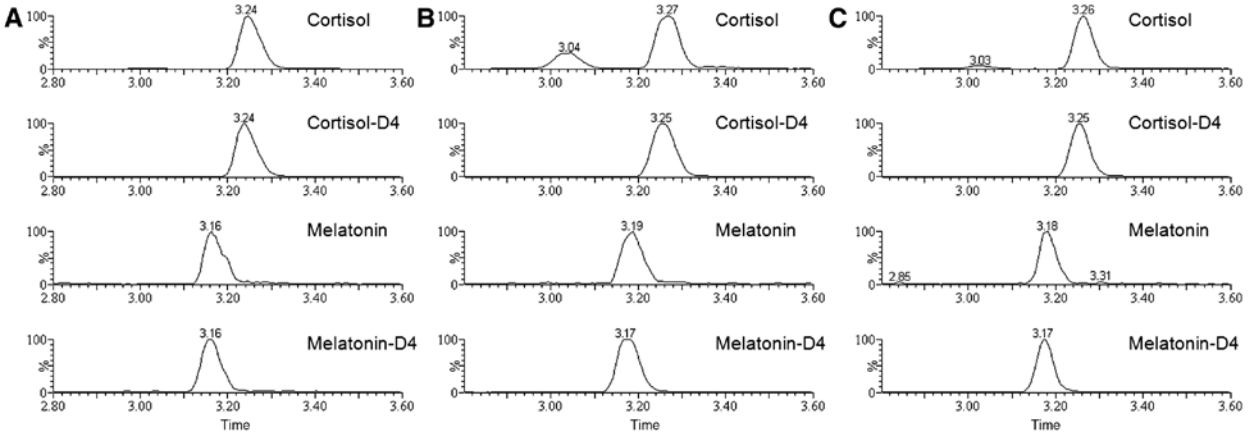

Figure 1: Representative chromatograms of cortisol and melatonin in plasma, saliva and plasma dialysate from the same individual. (A) Traces of the plasma sample (cortisol: 429 nmol/L; melatonin 84 pmol/L). (B) Traces of the saliva sample (cortisol: 8.4 nmol/L; melatonin 24 pmol/L) and panel C shows the traces of the plasma dialysate (cortisol: 23 nmol/L; melatonin 24 pmol/L).

Table 2: Intra- and inter-assay imprecision results for cortisol and melatonin in plasma and saliva.

	Plasma				Saliva			
	Intra-assay		Inter-assay		Intra-assay		Inter-assay	
	Mean	CV, %	Mean	CV, %	Mean	CV, %	Mean	CV, %
Cortisol, nmol/L								
Low	129	2.3	127	2.6	0.440	8.6	0.477	9.6
Medium	390	1.0	396	2.7	2.51	2.1	2.58	5.2
High	848	1.3	864	2.8	11.9	2.1	11.4	4.5
Melatonin, pmol/L								
Low	20.5	8.9	21.5	9.5	20.8	5.1	20.3	8.9
Medium	124	3.9	122	4.1	60.4	3.8	59.0	6.8
High	318	3.5	300	4.2	114	2.8	109	6.0

Table 3: Mean recovery results for cortisol and melatonin in plasma and saliva (ranges between parentheses).

	Plasma	Saliva
	Recovery	Recovery
	Mean (range), %	Mean (range), %
Cortisol, nmol/L		
Low	92.7 (86–97)	107 (98–116)
Medium	98.7 (96–103)	100 (95–104)
High	98.8 (96–101)	100 (95–105)
Melatonin, pmol/L		
Low	96.7 (86–107)	94.3 (87–105)
Medium	99.3 (92–107)	94.3 (87–111)
High	102 (91–111)	91.8 (85–112)

Three different samples (native concentrations in parentheses) were spiked at three different levels with respectively cortisol and melatonin.

for salivary cortisol (Figure 2A and C). The developed method compared well to the routine method with a slope of 1.02 (95% CI: 1.0–1.05) and an intercept of – 0.05 (95% CI: – 0.09 to 0.01). Bland Altman analysis showed a bias of 0.04 nmol/L between the two methods. For melatonin, we compared the developed method to an ELISA (Figure 2B and D). The developed method showed some discrepancy with the ELISA. In the lower range (< 30 pmol/L), there is reasonable agreement, but above > 30 pmol/L the ELISA measures considerably lower levels than our LC-MS/MS assay. Passing-Bablok regression gave a slope of 1.23 (95% CI: 1.09–1.51) and intercept of – 4.6. (95% CI: – 10 to 0.2). Bland Altman analysis showed a bias of 7.9 pmol/L between the two methods.

Impact of collection device and correlation between total plasma, free plasma and salivary melatonin and cortisol

For saliva melatonin and cortisol no significant concentration differences were observed between samples collected by passive drooling or Salivette in the 10 samples (see Figure 3A and 4A). Passing-Bablok regression gave the following equations, for melatonin: Salivette = 1.0 × passive drooling – 0.64; r = 0.98, for cortisol: Salivette = 0.999 × passive drooling – 0.65; r = 0.95. As there was no significant difference between saliva collected by passive drooling or Salivette, we used results of saliva obtained by passive drooling in calculations presented below.

Levels of total plasma cortisol correlated moderately with free plasma cortisol levels, r = 0.65 (Figure 3B). Free plasma cortisol levels correlated well with salivary cortisol levels, r = 0.87, but concentrations in saliva were approximately 50% lower compared to free plasma cortisol (Saliva = 0.51 × free plasma – 2.1; Figure 3C).

Total plasma melatonin correlated excellently with free plasma melatonin, r = 0.98; free plasma = 0.26 × total plasma + 1.2, indicating that approximately 75% of melatonin is protein-bound in plasma (Figure 4B). Comparison of free plasma melatonin with salivary melatonin showed that levels of melatonin in saliva were on average 36% higher when compared to levels of free plasma melatonin (95% CI = 4–60); passive drooling = 1.36 × free plasma – 1.1 (Figure 4C).

Discussion

In this study, we describe the characteristics, validation and application of a mass spectrometry based method for the simultaneous quantification of melatonin and cortisol in saliva and plasma. We demonstrated its potential by investigating the relationship between total plasma, free plasma and salivary melatonin and cortisol, and found that melatonin in saliva was on average 36% (95% CI: 4–60) higher in saliva than free plasma melatonin.

As melatonin and cortisol are frequently analyzed in circadian rhythm studies, combined analysis of these two neuroendocrine markers is a valuable asset. Quantification of these markers requires sensitive and accurate analytical methods that are routinely applicable. Despite the fact that immunoassays have provided a wealth of information on biochemical aspects of low molecular weight neuroendocrine biomarkers over the past decades, these techniques are rapidly being replaced by mass spectrometry based methods because of their superior accuracy [35]. This is especially true in the field of routinely applied biomarkers such as steroids, vitamins, and biogenic amines. In the field of chronobiology research still mainly relies on immunoassays, despite the fact that the limitations of these assays are becoming clear now. Melatonin is a (protein bound) low molecular weight biomarker that is present at very low concentrations amidst numerous potentially interfering structure analogs such as serotonin, N-acetylserotonin and kynurenamines. The same holds true for cortisol. This makes quantification of these markers by immunoassays vulnerable to variation in binding protein concentration, matrix and structure analog interferences [34, 36, 37].

Recently, two LC-MS/MS methods have been described in the literature for the combined analysis of cortisol and melatonin in saliva, but none for plasma [10, 11]. The two

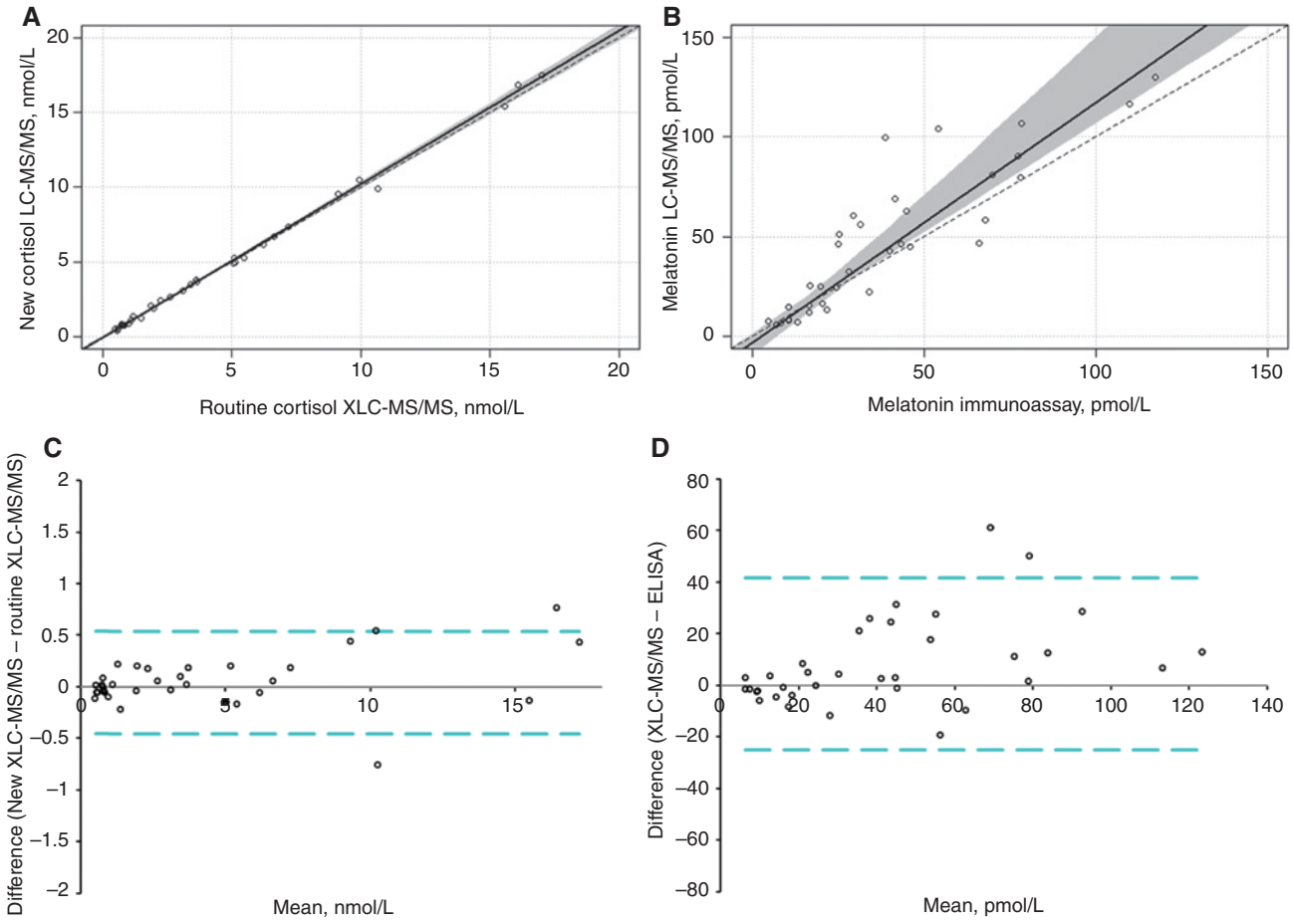

Figure 2: Scatter and Bland-Altman plots for the method comparison of saliva cortisol with XLCMS/MS (A, C) and saliva melatonin with commercial immunoassay (B, D). Solid line in the scatterplot (A, B) represents the line of identity.
Passing-Bablok regression analysis gave the following result for cortisol (A) $y = 1.02x - 0.05$ and for melatonin (B) $y = 1.23x - 4.61$. Gray area represents the 95% confidence interval. Bland-Altman plots are expressed with the method difference as absolute concentration (C, D). Dashed lines represent 95% limits of agreement.

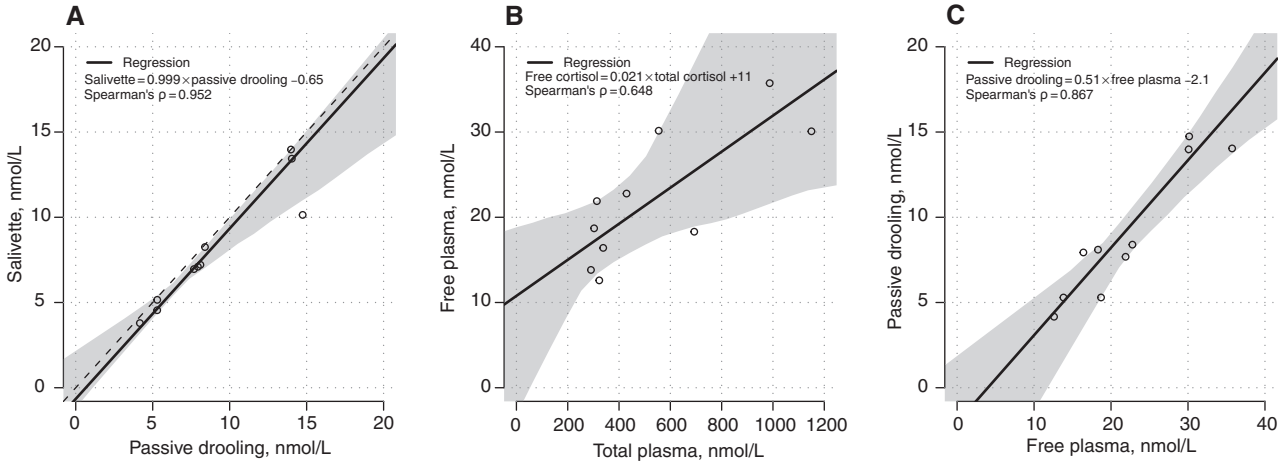

Figure 3: Cortisol: Correlation and Passing-Bablok regression between (A) passive drooling vs. Salivette, (B) total plasma vs. equilibrium dialysis, (C) free plasma vs. saliva.
Gray area represents the 95% confidence interval. Dotted line is the line of identity.

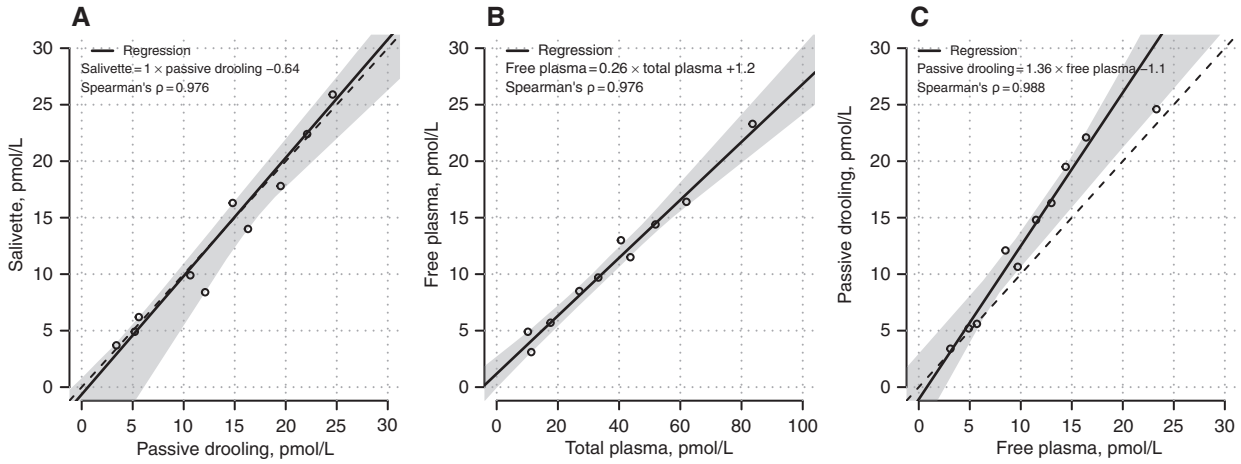

Figure 4: Melatonin: Correlation and Passing-Bablok regression between (A) passive drooling vs. Salivette, (B) total plasma vs. free plasma, (C) free plasma vs. saliva.
Gray area represents the 95% confidence interval. Dotted line is the line of identity.

described methods for saliva have certain drawbacks in comparison to our method, such as manual sample preparation (liquid-liquid extraction) [10] and a significantly higher LLOQ (0.5 nmol/L) for cortisol, which is insufficient to quantify late night saliva cortisol levels [11]. Moreover, in comparison to the other two published methods for cortisol and melatonin in saliva, our method is also suitable for plasma analysis. Our method enables efficient high throughput analyses of melatonin and cortisol, and was recently used in a large longitudinal study in which depressed and non-depressed individuals collected saliva over 30 days, thrice a day [38, 39].

In several studies in which immunoassays were used, it was found that the device for saliva collection can influence the results for melatonin and cortisol [40–42]. We therefore investigated to what extend sample collection methods influenced results of our LC-MS/MS assay. We found that results for melatonin and cortisol in saliva collected by either passive drooling or Salivette were similar. This finding is in line with previous results obtained by LC-MS [11]. A possible explanation for this observation could be that immunoassays generally are more sensitive to interfering substances as could be coming from the Salivette, whereas LC-MS/MS generally is less matrix-dependent [43]. We compared salivary melatonin results from our LC-MS/MS assay to a commercial immunoassay designed for use in saliva. The comparison showed reasonable agreement, with more scatter in the higher calibrator range. A difference in calibration between the melatonin immunoassay and our LC-MS/MS assay was excluded as explanation for the discrepancy, as calibrators were cross-checked (data not shown). The difference in results between IA and MS for salivary melatonin is in agreement with a previous publication from Jensen et al. which also reports a similar discrepancy between LC-MS/MS and an immunoassay [44].

In line with previous studies, we found that cortisol in saliva correlated well with plasma free cortisol, but not with total cortisol in plasma [45, 46]. Levels of cortisol in saliva were approximately half of that of plasma free cortisol. One contributing factor to this difference is probably the conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase in the salivary glands [47]. Levels of plasma free melatonin were 26% (95% CI: 22 –30%) of total melatonin in plasma, which corresponds well with values reported by Kennaway and Voultsios. (mean 23%, range 15–39%) [25]. However, salivary melatonin concentrations were on average 36% (95% CI: 4–60) higher than the corresponding plasma free melatonin concentrations, which could indicate local production of melatonin in the salivary glands. Recently, Shimozuma et al. reported the presence of the melatonin-synthesizing enzymes arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT) in epithelial cells of human submandibular glands [48]. Local synthesis of melatonin in salivary glands could explain the large variation found in the relation of salivary melatonin and plasma concentrations [49, 50]. This is to our knowledge the first study that investigated the relation between saliva and plasma dialysate by LC-MS/MS and shows a higher concentration of salivary melatonin vs. free plasma melatonin obtained by equilibrium dialysis. A limitation of this observation is that we only measured samples from 10 different individuals. To confirm local synthesis of melatonin in the salivary glands, more samples from different individuals should be included.

In summary, we showed that our mass spectrometry based method enables straightforward, reproducible and sensitive quantification of cortisol and melatonin in human saliva and plasma and illustrates the added value of reliable mass spectrometry based methods for the quantification of circadian rhythm biomarkers.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission. M.v.F. carried out all experimental work and performed the data analysis. I.P.K. made the concept and design of the study, interpretation of data, and drafted together with M.v.F. the manuscript. I.P.K. together with R.B revised and approved the final version of the manuscript.

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